REMARKS

Claim 62 reads as follows:

> 62. An isolated nucleic acid having a nucleotide sequence encoding an estrogen receptor β polypeptide comprising amino acids 1-45 of Figure 4, SEQ ID NO:2, wherein the polypeptide is capable of attenuating IL-1β-mediated NfkB transcriptional activation when contacted with estradiol in a cell.

Support for claim 62 is found at page 13, lines 9-13 of the instant specification. A copy of page 13 and accompanying Figure 7A is attached for your convenience.

Note that claim 62 is directed to an estrogen receptor β polypeptides. Such polypeptides were known in the art when this invention was made, and this term carried with it an accepted meaning with respect to structure and function.

The specification from page 10, line 17 to page 11, line 8 (copy attached), defines $hER\beta_L$ as an estrogen receptor expression vector resulting from cloning DNA encoding full-length hERβ into pDNA3 under the control of the CMV promoter. The new receptor can be expressed by having 530 residues, the first 45 of which are not found in the previously known human sequence (Figure 4) (see page 10, lines 21-23).

On page 8, lines 7-15 of the specification, (a copy is attached), $hER\beta_T$ is defined as the clone (designated R61010-2.24 or Clone 3) found to contain an insert with a nucleotide sequence identical to the published hERβ sequence (Mosselman et al., FEBS Letts. 392:49, 1996).

As noted on page 13, lines 10-13 of the specification, the results shown

in Figure 7 (copy attached) indicate that $hER\beta_L$ was capable of attenuating the IL-1β-mediated NFkB transcriptional activation, while $hER\beta_T$ exhibited no such inhibitory activity. As the specification points out, the only difference between $hER\beta_L$ and $hER\beta_T$ is the N-terminal 45 amino acids. This structural difference clearly affects function. Amino acids 1-45 are needed for the hERβ to attenuate 1L-1β-mediated NFkB transcriptional activation when contacted with estradiol in a cell, as claimed in claim 62. It bears repeating that claim 62 is directed to an estrogen receptor β.

In light of the foregoing, it is believed that claims 62-65 are fully supported by the specification , recite the patentable feature of the invention, and should be allowed.

Respectfully submitted,

Mary Elizabeth Brown
Reg. No. 46,579
Attorney for Applicants

DARBY & DARBY, P.C.
Post Office Box 5257
New York, NY 10150-5257
Phone (212) 527-7700

A fragment of approximately 1500 bp in length was produced. The fragment digested with *Hind*III and *Xba*I (which cleave at sites present in the forward and reverse primer sequences, respectively, but not in the main body of the amplified cDNA sequence) and cloned into the corresponding sites of the pcDNA3 expression vector (Invitrogen, Carlsbad CA). This assymetric cloning strategy places the 5' end of hERβ cDNA under the control of the viral CMV promoter in pcDNA3 (Figures 1 and 2). Several insert-containing pcDNA3 clones were identified. Plasmid DNA was prepared from three clones using a plasmid purification kit (Qiagen, Santa Clarita CA) and their insert sequences were determined by the dideoxy termination method. One clone (designated R61010-2.24 or Clone 3) was found to contain an insert with a nucleotide sequence identical to the published hERβ sequence (Mosselman et al., *FEBS Letts.* 392:49, 1996) and had the following 5' end structure:

M T G Y . . .
CCATC ATG ACC GGC TAT . . .

This clone was designated "truncated hERβ" or hERβ_T.

To verify the aminoterminal and upstream sequence of human hERβ, two independent approaches were taken, as described below.

(1) 10 μl of a human ovary 5'-Stretch cDNA library (Clontech, Palo Alto CA) was mixed with 50 μl of 1X K solution (1X PCR Buffer (GIBCO-BRL, Gaithersburg MD), 2.5 mM $MgCl_2$, 0.5% Tween-20, 100 μg/ml Proteinase K), and the reaction mixture was incubated at 56°C for 2 hours, then at 99°C for 10 minutes. 5 μl of this reaction mixture were then used as template in a nested PCR reation. For the first round, the forward primer (pDR2 sequencing primer, Clontech, Palo Alto CA) had the sequence 5'-

designated oligo #12908). The PCR reaction and cycling conditions were identical to those described in (1) above.

Excess nucleotides and primers were removed from the first round PCR reactions by purification over Wizard PCR columns (Promega). A second round PCR reaction was performed using 2 μl of the purified first round reaction. For the second round, the forward primer had the sequence 5'-ACTCACTATAGGGCTCGAGCGGC-3' (nested adaptor primer 2, Clontech), and the reverse primer had the sequence 5'-GTTGGCCACAACACATTTGGGCTTGT-3' (hERβ-specific, designated oligo #13871). The second round PCR reaction and cycling conditions were identical to those employed in the first round. The products were cloned into the pCR2.1 vector and two clones were sequenced. The two clones contain insert sequences of different lengths that are homologous to hERβ, to each other, and to the sequences isolated from a human ovary cDNA library as described above.

All of the hERβ sequences isolated by methods (1) and (2) above contained 110 nucleotides corresponding to hERβ_T sequences, as well as 228 additional nucleotides at the 5' end (Figure 3).

The hERβ cDNA sequence determined from these clones contained several important differences from the previously known human sequence. First, the third amino acid of the previous sequence was found to be F and not G (see above). Second, the methionine residue at the aminoterminus of the previous sequence was found not to be the initiator (i.e., true aminoterminal) residue. Rather, the authentic full-length hERβ cDNA sequence encodes a polypeptide having 530 residues, the first 45 of which are not found in the previously known human sequence (Figure 4). The sequence appears to be quite

homologous to rat ERβ; however, this reading frame was not identified previously (Kuiper et al., *Proc.Natl.Acad.Sci.USA* 93:5925, 1996). Furthermore, an optimal Kozak translation initiation sequence is found upstream of the newly discovered initiator methionine codon. A termination codon was identified 63 nucleotides upstream to the authentic ATG initiator codon in the same reading frame.

The cDNA encoding authentic full-length hERβ was cloned into pCDNA3 under the control of the CMV promoter; this expression vector was designated "long hERβ" or $hER\beta_L$.

Synthesis of full-length hERβ and truncated hERβ

To examine the natural start site for translation of hERβ, three plasmids were subjected to coupled transcription-translation, encoding $hER\beta_T$ (with a synthetic upstream translation initiation sequence), $hER\beta_L$ (with a synthetic upstream translation initiation sequence), and $hER\beta_L$ containing 93 nucleotides of its native upstream sequence (the entire sequence shown in Figure 3). The plasmids were transcribed and translated using the TNT T7-Coupled Reticulocyte Lysate System (Promega #L4610). Circular plasmid DNA was purified using Qiagen Maxi-Kit #12362. 2μg of the DNA was transcribed and translated in a single reaction in the presence of [^{35}S]-methionine (New England Nuclear, Boston MA). The translation products were resolved on a 10% SDS polyacrylamide gel and were visualized by autoradiography (Figure 5).

The resulting translation products of both $hER\beta_L$ products were of similar size (~63 kDa), and the $hER\beta_T$ product was appropriately shorter (~56 KDa). This indicates that the initiator ATG most likely utilized *in vivo* is the ATG at position 94-96. Utilization

(Tropix, Inc., Bedford MA); luciferase activity was then normalized to β-galactosidase activity.

The results shown in Figure 6A indicate that, in the presence of estradiol, hERβ$_T$ caused a 2-fold stimulation of ERE activity. By contrast, hERβ$_L$ under the same conditions caused a 6-fold stimulation of ERE activity. Thus, hERβ$_L$ is about 3-fold more active than hERβ$_T$ in this circumstance.

In a separate experiment, HepG2 cells were transfected with hERβ$_L$ or hERβ$_T$ as above, but the reporter gene consisted of three copies of an NFkB binding site upstream of the TK basal promoter. Transfected cells were incubated with or without interleukin-1β (IL-1β) to activate NFkB and/or with estradiol prior to luciferase determination. The results shown in Figure 7 indicate that hERβ$_L$ was capable of attenuating the IL-1β-mediated NFkB transcriptional activation (to an extent similar to that observed with hERα), while hERβ$_T$ exhibited no inhibitory activity.

(ii) Human endothelial cells:

HAECT-1 cells (a clonal immortalized human aortic endothelia cell line derived by infection with Ad5 ori-SV40 ts A209) were transfected with pcDNA3hERβ$_T$ or pcDNA3-hERβ$_L$ and ERE-luciferase plasmids by electroporation. After 4 hours, the cells were treated overnight with or without 100 nM 17-β estradiol prior to luciferase activity measurements. The results shown in Figure 8 indicate that hERβ$_L$ is 2-3 times more active than hERβ$_T$ in activating the ERE-reporter gene in the presence of estradiol. In independent experiments, cells transfected under identical conditions were monitored for their levels of estrogen receptors using a ligand binding assay. The results indicate that the increased activity of hERβ$_L$ relative to hERβ$_T$ is not due to an increase in receptor number or stability,